Huntingtin-associated protein 1: A potential biomarker of breast cancer

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Abstract. It is reported that patients with Huntington’s disease (HD) have a low incidence of cancer. In this study, we investigated the expression of huntingtin-associated protein 1 (HAP1), the ligand of HD's production, in breast tumor and normal tissues. We found that HAP1 expression was significantly lower in tumor compared to normal tissues. We then transfected the HAP1 gene into the breast cancer lines MCF-7 and MDA-MB-231, and results showed that the overexpression of HAP1 reduced the growth of the two cell lines. In addition, we observed that HAP1 also reduced invasion and migration, and upregulated apoptosis in MCF-7 cells; however, these changes were not observed in MDA-MB-231 cells. We also demonstrated that the expression of EGFR and apoptosis-related genes might be involved in cell proliferation and apoptosis. In conclusion, overexpression of HAP1 reduced in vitro cell growth in breast cancer cell lines, suppressed the migration and invasion, and promoted the apoptosis of certain cell lines. Therefore, HAP1 is a potential molecular target for the diagnosis and treatment of breast cancer.

Materials and methods

Introduction

Breast cancer is the most prevalent tumor in women, and its incidence accounts for 7-10% of all malignant tumors. Huntington's disease (HD) is an autosomal dominant hereditary neurodegenerative disorder characterized by late onset, progressive psychiatric disruption, cognitive deficits and loss of motor coordination (1). According to a previous study, the overall incidence of cancer is significantly lower among patients with HD, and this seems to be related to intrinsic biologic factors (2). HD is caused by a CAG triplet repeat expansion in exon 1 of the huntingtin (Htt) gene, encoding an abnormal expanded polyglutamine (polyQ) tract that confers toxicity to the mutant Htt protein. When the mutant Htt gradually accumulates in the cell, it can affect the function of several proteins and the conduction of some signaling pathways. Huntington-interacting protein 1 (HIP1) and huntingtin-associated protein 1 (HAP1) are two known ligands of Htt (3,4). Research on the relevance between HIP1 and cancer showed that HIP1 had a high expression level in breast, colon, prostate and other types of cancer (5,6). However, there are currently no studies regarding the association between HAP1 and cancer. In this study, we first examined the level of HAP1 in human breast tumor and normal breast tissues, and investigated the roles and possible mechanisms of HAP1 in human breast cancer cells. In addition, we selected ER positive MCF-7 and triple negative MDA-MB-231 cells, representing two main types of breast cancer, for research.

Tumor samples. A total of 43 breast carcinoma specimens with non-neoplastic adjacent tissues from patients with primary breast tumor were collected during the surgical procedures at the Department of General Surgery, Jiangsu Cancer Hospital, Nanjing Medical University (Nanjing, China). The sample collection was carried out in accordance with the National Regulation of Clinical Sampling in China.

Cell culture. The human breast cancer cell lines MCF-7 and MDA-MB-231 were maintained in DMEM with high glucose (Gibco, Grand Island, NY, USA) and 10% FBS (Gibco).

Viral production and infection of target cells. Retrovirus was generated by cotransfection of pBabe-puro empty vector (as control) or pBabe-puro-Hap1 plasmid along with pVSV-G (envelope) and pVSV-GP (packaging) plasmids in 293FT cells, growing at 85-90% confluency in 10-cm petri dishes. After 48 h, the culture medium containing the viral particles was harvested. Target cells were infected with virus containing medium in the presence of 10 µg/ml polybrene (Sigma, St. Louis, MO, USA). Then, changing back to fresh medium, cells were selected with 2 µg/ml puromycin (Fischer, USA) for 7-10 days, re-cultured in a larger dish containing puromycin.
and used for further assay. All plasmids and cells were kindly provided by Professor Jinrong Zhou, at Harvard University (Cambridge, MA, USA).

**Cell viability assay.** Cells (3,000/well) were plated in 96-well plates. Each group had five replicates. The growth was monitored 72 h later and cell viability was measured by CCK-8 (Dojindo, Kumamoto Prefecture, Kyushu, Japan).

**Colony formation assay.** Cells were seeded in six-well culture plates at 200 cells/well. After 10 days of incubation, the cells were stained with 0.1% crystal violet (Enox, Shanghai, China). The colonies with >50 cells were counted. The colony formation rate was acquired through colony numbers/total seeding cells.

**Semiquantitative RT-PCR.** Total RNA was extracted with the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and was then reverse transcribed using PrimeScript RT Master mix (Takara, Kumamoto Prefecture, Kyushu, Japan). Real-time PCR was performed using an ABI 7300 Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) with the SYBR Premix Ex Taq (Takara). All samples were analyzed in triplicate and in optically clear 96-well plates (Corning Inc., Corning, NY, USA). The cycling parameters were: 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec, 60°C for 31 sec and 95°C for 15 sec, 60°C for 60 sec, 95°C for 15 sec. The human β-actin transcript was used as an internal reference to control for variations in the total mRNA quantity of each sample. Each RNA sample was analyzed in triplicate. Primer sequences are listed in Table Ⅰ.

**Western blot analysis.** Cells were collected and lysed with lysis buffer (Beyotime, Jiangsu, China), and concentrated to obtain the proteins. The amount of total proteins was estimated by OneDrop OD-1000+ Spectrophotometer (OneDrop, Shanghai, China), and then proteins were mixed with SDS-PAGE buffer (Beyotime) and boiled for 5 min. The proteins were loaded onto 10% SDS-PAGE gel and, following electrophoresis, the proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Then, the membrane was blocked with nonfat-dried milk, incubated with primary and secondary antibody (Dako, Japan), and protein bands were visualized by ECL detection reagent (Millipore, Billerica, MA, USA).

**In vitro migration and invasion assays.** For the migration assays, the cells were detached and aliquots of 2x10⁵ cells/ml were plated onto the inserts of the 8-µm pore-sized Transwell chambers (Corning Inc.). For the Transwell invasion assays, 1x10⁶ cells were plated onto inserts containing a polycarbonate membrane with a thin layer of BD Matrigel Matrix (BD Biosciences, Franklin Lakes, NJ, USA). Both migration and invasion were assayed 24 h later by counting the cell numbers across the membrane.

**Flow cytometric assay.** For cell apoptosis, a total of 1x10⁶ cells were transferred to a tube in which 5 µl Annexin V and 1 µl propidium iodide (PI) were added after being resuspended with 100 µl binding buffer (Invitrogen). The cells were then allowed to incubate at room temperature for 15 min and were analyzed using flow cytometry. For cell cycle analysis, cells were collected, washed twice with PBS, supplemented with 1 ml 75% ethanol, and kept at -20°C overnight. The cells were then resuspended with PBS, supplemented with PI and RNaseA, incubated for 30 min at 37°C and analyzed using flow cytometry.

**Statistical analyses.** Data are expressed as the means ± SD, and statistical significance was assessed by Student’s t-test and one-way ANOVA. P<0.05 was considered to indicate a statistically significant difference.

### Results

**Expression of HAP1 in breast tumor samples.** HAP1 expression was assessed by qRT-PCR in surgical specimens from patients with breast cancer. As shown in Fig. 1, HAP1 expression was markedly reduced in most cancer tissues compared with normal breast tissues. Of 43 cases, ~70% showed HAP1 expression levels in normal breast tissues >3-fold higher than those of matched tumors (Fig. 1).

**Expression of HAP1 in transfected and control cells.** The MCF-7 and MDA-MB-231 cells transfected with pBabe-puro-HAP1
and pBabe-puro were labeled MCF-7/HAP1, MCF-7/pBabe and MDA-MB-231/HAP1, MDA-MB-231/pBabe, respectively. Western blotting and qRT-PCR results of different cells showed that MCF-7/HAP1 (P<0.006) and MDA-MB-231/HAP1 (P=0.001) had a significantly higher expression of HAP1 than their control groups. Moreover, there was no obvious difference between pBabe and negative control groups (P>0.05) (Fig. 2A and B).

**Effects of HAP1 on breast cancer cell growth and expression of EGFR in cells.** At 72 h after cells were plated in 96-well plates, upregulation of HAP1 significantly increased the cell viability of both HAP1 overexpression cells in MCF-7 [HAP1 mean, 76.0±6.8 vs. pBabe mean, 94.0±6.6 (P=0.03) vs. control mean, 100±0.0 (P=0.004)] and MDA-MB-231 [HAP1 mean, 68.2±17.4 vs. pBabe mean, 99.8±6.0 (P=0.041) vs. control mean, 100±0.0 (P=0.034)] compared with empty vector plasmid transfected cells and negative control cells (Fig. 3A). In addition, colony formation rates were significantly decreased in MCF-7 cells with HAP1 overexpression [HAP1 mean, 22.7±1.3% vs. pBabe mean, 33.8±0.8% (P=0.000) vs. control mean, 35±0.5% (P=0.000)] and MDA-MB-231 [HAP1 mean, 9.3±1.9% vs. pBabe mean, 15.8±2.5% (P=0.022) vs. control mean, 16±2.6% (P=0.024)] when compared with those of pBabe and control cells (Fig. 3B). We also compared the expression of EGFR in three groups of the cell lines. We observed that in the MCF-7 cell lines, EGFR expression of MCF-7/HAP1 [HAP1 mean, 7.0±0.4 vs. pBabe mean, 1.0±0.1 (P=0.000) vs. control mean, 1.0±0.0 (P=0.000)] was ~7-fold higher than that of the other two groups. However, there was no difference in EGFR expression between the three groups in the MDA-MB-231 cell lines (F=3.2; P=0.1) (Fig. 3C).
Overexpression of HAP1 impairs migration and invasion. We observed that MCF-7/HAP1 had fewer cells across the membrane than MCF-7/pBabe and MCF-7 in migration assays [HAP1 mean, 50.0±3.6 vs. pBabe mean, 271±9.5 (P=0.000) vs. control mean, 261±4.6 (P=0.000)] (Fig. 4A and B). In addition, compared to the number of the other two cell groups, the number of MCF-7/HAP1 cells that invaded the Matrigel-coated filter was significantly lower [HAP1 mean, 3.3±0.6 vs. pBabe mean, 13.3±1.5 (P=0.000) vs. control mean, 10.3±1.5 (P=0.000)] (Fig. 4A and C). Thus, the overexpression of HAP1 in MCF-7 can curb tumor cell migration and invasion. However, in MDA-MB-231 cells, there was no significant difference in the number of cells across the membrane in both cell migration (F=0.8; P=0.5) and invasion (F=0.3; P=0.7) (Fig. 4A-C).

Effects of HAP1 on cell cycle, apoptosis and expression of relative genes. According to cell cycle distribution analysis by flow cytometry, HAP1 increased the percentage of cells in the G2-M phase. In MCF-7 cells, the percentage in the G2-M phase of the HAP1 group was 30.84%, higher than the 13.17% and the 10.61% in the pBabe and control groups. In MDA-MB-231 cells, the percentage in the G2-M phase of the HAP1 group was 14.84%, also higher than the other two groups (Table II). The results indicated that HAP1 induced G2-M arrest in both...
Furthermore, \textit{HAPI} overexpression significantly induced apoptosis of MCF-7 cells compared with pBabe and control groups, and there was also no significant difference in MDA-MB-231 cells (Fig. 5A). Therefore, we detected the expression of genes, including caspase-3, -8, -9, Bcl-2, Bax and survivin, involved in cell apoptosis and survival, by RT-PCR (Fig. 5B and C). The results showed that the expression of caspase-3, -9, Bcl-2, Bax and survivin in MCF-7/HAPI was

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<th>MCF-7</th>
<th>MDA-MB-231</th>
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<tr>
<td></td>
<td>HAPI</td>
<td>pBabe</td>
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<tr>
<td>G0/G1 (%)</td>
<td>36.53±0.96</td>
<td>46.04±2.22</td>
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<td>S (%)</td>
<td>32.63±0.34</td>
<td>40.78±0.41</td>
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<tr>
<td>G2/M (%)</td>
<td>30.84±1.09</td>
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*P<0.05.
significantly higher than in the other two groups. Moreover, the expression of caspase-3 was $>800$ times higher in MCF-7/HAPI compared with the other two groups [HAPI mean, 857.7±278.9 vs. pBabe mean, 1.0±0.1 (P=0.000) vs. control mean, 1.0±0.0 (P=0.000)], and it was confirmed by western blotting (Fig. 5D).

**Discussion**

HAPI is a type of cytoplasmic protein distributed mainly in the microtubules and membranous organelles, including the mitochondria, endoplasmic reticulum, tubulovesicles, endosomal and lysosomal organelles, and synaptic vesicles (7.8). HAPI affects the synthesis of certain proteins and the conduction of signals (9-11). As a ligand of the production of HD, it was initially studied in the nervous system, whereas later research showed that it was involved in the other systems.

Herein, we detected the expression of HAPI in breast tumor and normal breast tissues. In contrast to normal breast tissues from breast cancer patients, we observed that HAPI expression was reduced in the majority of breast tumor samples. To the best of our knowledge, this is the first published study to show that HAPI is downregulated in breast tumor tissues and, based on these findings, it is suggested that HAPI plays a role in the pathogenesis of breast cancer.

HAPI is involved in numerous cellular functions, including cell proliferation and apoptosis (12,13). In this study, upregulation of HAPI resulted in a significant decrease in the cell growth and colony formation rate of both breast cancer cells. Furthermore, cell cycle results showed that the percentage of G2/M phase increased in both MCF-7 and MDA-MB-231 cells, indicating that cell growth could be arrested at the G2/M phase by HAPI. Moreover, apoptosis may be involved in the inhibition of cell growth. It has been reported that apoptosis plays an important role during the malignant transformation of normal cells. The above studies, along with ours, indicate that loss or downregulation of HAPI may disrupt the balance between proliferation and apoptosis and may represent a key pathogenic step in the development of breast cancer. The cell apoptosis assay confirmed that HAPI induced cell apoptosis and increased expression of relative genes in MCF-7 cells, particularly caspase-3. Caspase-3 is located at the downstream of the apoptosis signaling pathway, and plays a key role in cell apoptosis (14,15). Collectively, HAPI-induced apoptosis in MCF-7 cell lines may be mainly mediated by the caspase-dependent pathway; in the MDA-MB-231 cells, however, the three groups showed no difference in the expression of those genes.

Previous studies showed that inhibition of HAPI expression decreased EGFR signaling and cell viability, whereas overexpression of HAPI enhanced this signaling activity (12). EGFR is expressed in various types of tissue, including epithelial, mesenchymal and of neuronal origin, and plays a major role in normal cellular processes such as proliferation, differentiation and development. EGFR is also highly expressed in a number of solid tumors and its expression correlates with tumor progression, resistance to chemotherapy and a poor prognosis (16,17). Our detection of EGFR showed that EGFR expression was higher in MCF-7/HAPI, which negatively correlated with the results of cell proliferation. Therefore, we considered that EGFR might play a role in MCF/HAPI, although not a major one; on the contrary, the effect of EGFR was counteracted by others.

Although it is unclear why the effect of HAPI is significant in MCF-7 cells and not in MDA-MB-231 cells, we propose a hypothesis (Fig. 6). It was reported that more than 90% sporadically lurking HAPI-immunoreactive (HAPI-ir) cells expressed nuclear estrogen receptor (ER) (18). Therefore, we hypothesized that ER-positive MCF-7 cells gathered more HAPI than triple negative MDA-MB-231 cells and interacted with them.

In various cells, the Ca$^{2+}$ signaling process is mediated by the endoplasmic-reticulum-membrane-associated 1,4,5-trisphosphate (Insp$_R$) receptor (Insp$_R$), which has a critical role in the control of cellular and physiological processes as diverse as cell division, cell proliferation and apoptosis (19,21). Tang et al found that Insp$_R$ could bind with HAPIA in vitro and in vivo, suggesting that HAPI may impact the function of Insp$_R$ (20). When Insp$_R$ is activated by HAPI, the Ca$^{2+}$ release is increased, subsequently inducing the activation of calpains.

The calpains are a family of neutral cysteine proteases that require calcium for their catalytic activity. The cellular activity of calpain is, in part, regulated by their endogenous inhibitor calpastatin (22,23). The calpains function in the controlled proteolysis of a large number of specific substrates involved in various cellular processes such as migration, cell signaling and apoptosis. In addition, previous studies showed that calpain cleaved caspase-9 and therefore blocked the caspase-3 activation (24). Furthermore, Storr et al suggested that ER-positive breast cancer had a significantly high calpastatin expression compared with ER-negative breast cancer, and the expression of calpastatin in non-triple negative breast cancer was also significantly higher than in triple-negative breast cancer (22). The expression of calpain 1, however, was the opposite of calpastatin. Based on these data, we deduced that in ER-positive or (and) non-triple negative breast cancers, more apoptosis was induced than in ER-negative or (and) triple-negative breast cancer.
Collectively, ER, expressed in MCF-7, induced HAP1 assembly, then sequentially activated Insp,R, calpastatin and caspase-3, finally increasing apoptosis. Moreover, the procedure may also impact invasion. This pathway does not exist in MDA-MB-231. However, further research is required to verify the hypothesis and to clarify the mechanism.

In summary, our findings demonstrated for the first time that HAPI downregulation occurs in breast tumor, and that HAPI suppresses breast cancer cell growth. However, there were also some different effects of HAPI between ER-positive MCF-7 and triple negative MDA-MB-231 cells, which we hypothesize may reflect the different types of breast cancer in the clinic. The findings of this study may provide a new therapeutic target for the treatment of breast cancer patients.

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References